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A Humidity-induced Convective Throughflow Ventilation System Benefits *Annona squamosa* L. Explants and Coconut Calloid

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A simple apparatus is described for generating pressurized throughflow ventilation in plant tissue culture vessels. No pumps or gas-cylinders are required and the flow is driven by humidity-induced diffusion across microporous membranes. In the experiments described, pressurized flows of sterile humidified air were supplied at rates of up to 1 ml min^{-1} and these had beneficial effects on leaf survival and production in *Annona* cuttings and on calloid form in coconut. Ethylene (ethene) was removed more quickly from the pressure-flow ventilated culture vessels (t_{50}, 0·4–0·7 h) than from those aerated by diffusion through conventional polypropylene membranes (t_{50}, 1·6–2·4 h).

In *Annona* cuttings leaf production was greatly increased and ethylene-induced leaf fall considerably delayed when cultured with the forced as opposed to diffusion-based ventilation of the vessels.

With throughflow ventilation, coconut calloid was more convoluted than under wholly diffusive aeration and had a smooth distinct epidermal surface and clearly defined sub-epidermal meristematic nodules. It resembled freshly initiated calloid from which regeneration of plantlets via somatic embryogenesis can be obtained. Under wholly diffusive aeration, calloid developed a rough, relatively undifferentiated surface, more haustorial (i.e. cotyledonary) in appearance, and characteristic of cultures where regeneration potential has been lost.

It is suggested that other benefits of the pressurized throughflow ventilation may be the removal of volatiles such as ethanol and acetaldehyde, the removal of excess carbon dioxide at night and its improved supply during the day, and sustained oxygen concentrations at levels close to atmospheric both night and day.

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Key words: Abscission, callus, ethylene, explants, tissue-culture, growth, ventilation.

INTRODUCTION

Widespread concern has been expressed recently regarding the adequacy of the ventilation achieved in some conventional plant tissue-culture systems (Woltering, 1986; Jackson *et al*., 1987; Adkins, 1992; Debergh *et al*., 1992; Righetti and Facini, 1992; Rossetto, Dixon and Bunn, 1992). Current methods for ventilating callus, explants and cuttings in vessels under sterile conditions often rely upon the diffusion of gases through microporous membranes, or gas-permeable films, at the mouth or in the wall of the vessel. Alternatively, in sealed vessels, aeration may be dependent upon photosynthetically generated oxygen. Culturing in diffusive ventilation or in sealed vessels, although preventing the contamination of the culture by air-borne bacteria or fungal spores, can lead to the accumulation of acetaldehyde, ethylene (ethene) and ethanol (Thomas, Des and Murashige, 1979; Righetti, Magnanini and Maccaferri, 1987; Righetti, Magnanini and Infante, 1990), while concentrations of carbon dioxide and oxygen may be lowered or raised from their physiological optima (De Proft, Maene and Debergh, 1985). Some species are especially susceptible to ethylene accumulation. For example, Jackson *et al*. (1991) found that *Ficus lyrata* and *Solanum tuberosum* cv. Red Craig's Royal (potato) were adversely affected by poor aeration in tightly sealed culture vessels. After 28 d, the leaf area of *Ficus* was reduced by more than 50%, while shoot height in *Solanum* was reduced by 64% after 14 d, and leaf expansion was minimal. Jackson *et al*. (1991) concluded that accumulated ethylene was responsible for these effects, since in both species the use of an ethylene absorbent or antagonist in the tightly sealed vessels led to normal growth, indistinguishable from that in loosely sealed vessels where the endogenously generated ethylene could escape easily.

The benefits of enhanced diffusive ventilation for *Malus domestica* seedlings as well as potato explants were emphasized in a subsequent paper (Jackson, Belcher and Brain, 1994). Also, Rossetto *et al*. (1992) found that improved diffusive aeration of tissue-cultured rare Australian plants, including species of *Conostylus*, *Diplolena*, *Drummondita*, *Lechenaultia*, and *Sowerbaea*, reduced vitrification and significantly improved shoot quality and the later establishment of plants in soil. Buddendorf-Joosten and Woltering (1996) have reported on an improved diffusion-based ventilating system for potato plantlets in which the atmosphere surrounding the culture vessels was constantly refreshed and humidity-controlled.

An alternative to diffusive ventilation is to use forced ventilation of the culture vessels, and it is becoming increasingly apparent that forced ventilation can have a number of advantages over conventionally sealed systems or those relying on gaseous diffusion (Kozai, Kitaya and

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Kubota, 1995): growth of cuttings, seedlings and callus can all be improved. For example, Kozai, Kubota and Nakayama (1989) found higher photosynthetic rates and growth of strawberrry plants *in vitro* with forced ventilation than with conventional *in vitro* conditions, as did Yue, Gosselin and Desjardins (1993) using *Pelargonium* cuttings. Adkins (1992) found that rice callus benefitted from a forced ventilation of the head-space of the tissue culture vessels and attributed the improved performance to ethylene removal and an improved oxygen regime. However, the systems used to achieve forced ventilation are usually rather complex and require compressed gases or electrically-driven pumps, filtration systems, and often gas-mixing/metering devices to maintain the flow balance to the culture vessels.

In this paper we describe an alternative non-mechanized apparatus for achieving forced ventilation. The apparatus delivers a sustained stream of sterile humidified air driven by the process of humidity-induced diffusion. This process, which depends only upon the maintenance of a water vapour gradient across a microporous membrane, is also a major cause of pressurized gas-flows in a number of wetland and aquatic macrophytes (Dacey, 1981; Armstrong and Armstrong, 1990; Armstrong *et al*., 1990; Brix, Sorrell and Orr, 1992).

We report on the effects of the pressurized ventilation on the growth of explants of the ethylene-sensitive species *Annona squamosa* L. (Lemos and Blake, 1994), and on the growth and differentiation of the recalcitrant calloid of coconut. In the case of *Annona*, the culture medium was prepared either with or without an ethylene antagonist. The system is compared with conventional diffusive ventilation of tissue cultures using polypropylene membranes; also, the rates of ethylene efflux from, and its accumulation within, culture vessels ventilated in various ways are reported.

THE PRESSURE-FLOW VENTILATING APPARATUS AND THE UNDERLYING PRINCIPLES INVOLVED

Humidity-induced pressure flow (convective throughflow) in the wetland plant *Phragmites australis* (Cav.) Trin. ex Steud., is driven by a diffusion of oxygen and nitrogen through the stomata of the leaf sheaths into the humid atmosphere of the substomatal cavities (Armstrong and Armstrong, 1990). Here, the constant humidification of the internal atmosphere (up to 2–3% by volume) creates and maintains water vapour levels, thus diluting the atmospheric gases oxygen and nitrogen and producing a concentration gradient for their inward diffusion from the drier outer air. If stomatal resistance to inward diffusion is effectively less than any Poiseuille resistance to backflow to the atmosphere, the inwardly diffusing air will cause a pressurization within the plant. If there was no other path for gases to escape, the total pressure within the leaf sheath gas space at equilibrium would be greater than atmospheric by an amount numerically equal to the water vapour partial pressure beneath the stomata. In plants such as *Phragmites*, however, there is an alternative outflow path, and the tendency to pressurize drives a convective flow (pressure-flow) of gases into the underground parts and back to the atmosphere through old dead flowering shoots—hence the term convective throughflow.

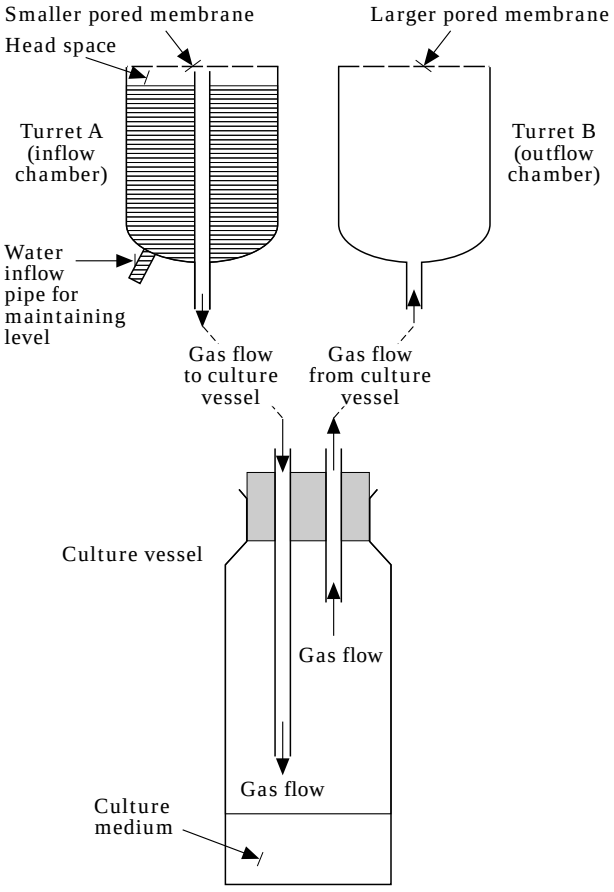

FIG. 1. Pressurized ventilating apparatus for continuous supply of humidified sterile air to the head-space of tissue culture vessels; the pressure-flow mechanism depends upon humidity-induced diffusion of atmospheric air into turret A through small-pored Nuclepore membrane (pore diameters 0·03 μm). Venting is through the larger-pored membrane (pore diameters 0·2 μm) on turret B. A water-filled syringe permanently attached to the water-inflow pipe may be used for maintaining the water level in Turret A. In this example the gas-flow into and from the culture vessel is via glass tubes.

It was envisaged that a simple ventilation system based on the principle of the humidity-induced convective throughflow found in *Phragmites* could be devised to provide a sterile flow of air to tissue culture vessels if two chambers, each bearing a microporous membrane, were combined. A smaller-pored, humidified inflow chamber would induce the flow, while venting would take place via a dry outlet chamber. In the humidity-induced pressure-flow ventilating apparatus described here (Fig. 1), the pressurization and sterile humidified gas-flow was created using microporous Nuclepore membranes (pore diameters 0.03 μm; thickness 10 μm; membrane porosity approx. 10%) overlying, but not touching, a reservoir of water in a glass 'inflow turret' (turret A: internal diameter, 20 mm; length, 30 mm). A glass 'outflow tube' opening into the humidified head-space of turret A, just below the membrane, directs the incoming gases to the header space of the culture vessel. Venting to the atmosphere occurs through a second, 'dry' turret (outlet

turret B) capped by a larger pored Nuclepore or Millipore membrane (pore diameter, 0·2 μm; porosity and thickness as for turret A). This pore size allows the gases to be vented easily under the pressure differentials generated by the inflow turret, and maintains the sterility of the apparatus. A syringe containing sterile water for filling the turret and adjusting the water level is connected to a side-arm on turret A.

A patent for this type of apparatus (No. GB227505B–UK Patents Office) has recently been granted (Armstrong and Armstrong, 1996). Details of the functioning of Nuclepore membranes for inducing pressurizations and gas-flows are to be found in Armstrong (1992), Armstrong and Armstrong (1994) and Armstrong, Armstrong and Beckett (1996*a*, *b*).

MATERIALS AND METHODS

Plants were grown in glass culture tubes, (length 75 mm, i.d. 23 mm, capacity 30 ml), fitted with either, (*a*) a conventional diffusive ventilator consisting of a disc of polypropylene film (thickness 25 μm: oxygen transmission rate, $< 1{\cdot}8 \times 10^{-2}$ m^3 m^{-2} d^{-1} MPa^{-1}: Courtaulds Films, Bridgewater, Somerset, UK), secured over the mouth of the tube by a rubber band, or (*b*) the humidity-induced pressure-flow ventilating system (Fig.1). The reservoir water level in the pressure-flow assembly was raised initially to within 1–2 mm of the membrane each day, but since the water level tended to fall by 1–4 mm during 24 h, it was re-adjusted to the original level at the beginning of each day. For throughflow ventilation, the culture tube was fitted either with a 'Suba-seal' cap penetrated by two hypodermic needles to serve as outflow and inflow paths to and from the turrets, or with a rubber bung and two glass tubes, the longer tube being for the inflow. Each of these tubes was connected to a turret by silicone rubber tubing of wall thickness 1·4 mm (Fig. 1).

Under the conditions of most of the experiments: 25–29 °C and 40–60 % relative humidity (RH), the ventilation rate for a throughflow system, starting with the water close to the membrane (1–2 mm below) at the beginning of the day, ranged between 0·5 and 1·0 ml min^{-1}. Thus, at the fastest flow rate, the air could be 'renewed' approximately every 30 min in a 30 ml culture tube.

Throughflow ventilation rates

Various procedures were used to characterize the functioning of the assembled pressurized ventilating system. Firstly, gas flows within the assembly were measured using a soap film flow meter joined in series with the down-pipe from the inflow turret to the culture vessel. Flow measurements were made with culture vessels (*a*) empty, (*b*) containing 5 ml agar, (*c*) containing 5 ml agar together with a leafy cutting, and (*d*) with the culture vessel empty and the inflow turret A dry. Growth room conditions were 25 °C and 17 % RH, and there were four replicates per treatment.

Secondly, flows were measured with and without an outflow turret and with the distance between the water surface and inflow membrane varied between 2 and 19 mm. Growth room conditions were 21·5 °C and 36 % RH, and there were four replicates per treatment. Here the culture vessels were empty.

Ventilating efficiencies—t_{50} measurements using ethylene

The efficiency of ventilating systems can be compared by measuring the time taken, (t_{50}), for half of an injected standard sample of a marker gas, e.g. ethylene, to escape from the vessel (Jackson *et al.*, 1991). The t_{50}s were measured with the culture vessels capped in various ways, using: (*a*) 'Suba-seal' rubber puncture caps, (*b*) cling-film PVC (thickness, 10 μm), (*c*) conventional polypropylene discs (thickness, 25 μm), (*d*) the convective ventilation apparatus with hypodermic needles, (*e*) the convective ventilation apparatus with glass tubing, and (*f*) as for (*e*), but with inflow turret A dry. Two sets of comparisons were made, the first (set A) involved systems a–e, the second (set B) involved only c, e and f.

For set A, the vessels were open ended tubes, similar in size to the culture tubes, and were 'capped' at the top as described above and at the bottom with a 'Suba-seal' rubber puncture cap; gas samples were added or withdrawn through this using a hypodermic syringe. At each addition or removal of gas, an equal volume of chamber-gas or air was respectively removed or added to maintain a constant pressure in the assembly. Five of each type of assembly were used as replicates, and 0·21 ml volumes of 1 % ethylene in nitrogen were injected through the lower 'Suba-seals' sufficient to create an initital concentration of ethylene in each assembly of 70 μl l^{-1}. Ethylene concentrations were then determined at regular intervals by removing 0·5 ml samples of gas from the assemblies and analysing by means of gas chromatography (Vega 6000-GC, Carlo Erba Strumentazione). Porapack (80–100 mesh) was used in a stainless steel column (800 × 1·5 mm), and column, injector and flame ionization detector temperatures were 80, 150 and 150 °C, respectively. Nitrogen was used as the carrier gas at a rate of 50 ml min^{-1}. The time taken (t_{50}) for the escape of 50 % of the ethylene was determined from plots of ethylene concentration against time. Growth room conditions were 25 °C and 40–60 % RH, and the inflow membrane pore diameters were 0·03 μm.

For set B normal culture vessels were used, but each had a side arm with a 'Suba-seal' for adding or removing gas samples. Vessels were initially filled with 23 μl l^{-1} ethylene and 500 μl samples were removed for analysis at various time intervals depending upon the ventilation system being tested. However, each vessel was sampled only once to avoid artificially diluting the sample. To this end nine vessels per treatment were used for each run, and the runs were replicated four times. The ethylene samples were analysed by GC (Pye Unicam, Model GCD) using Poropack Q (60–80 mesh) in a glass column (2500 × 6·5 mm) at 100, 150 and 150 °C. The peak (which was removable by pre-exposure of samples to permanganate) was identified by a retention time of about 1·4 min and nitrogen was used as carrier gas at a rate of 60 ml min^{-1}. The ventilating assemblies were in a growth room at 27 °C and 25 % RH.

Growth of Annona *explants with diffusive or convective ventilation and with or without an ethylene antagonist*

Annona nodal cuttings of 20 mm, from new fluxes of mature plants previously grown in a glass house, were transferred, one to each culture tube containing 8 ml of MS (Murashige and Skoog, 1962) medium with 20 mg l^{-1} sucrose, 2 mg l^{-1} BAP, 3 g l^{-1} Phytagel and pH corrected to 5·8 prior to autoclaving.

Replicates (four per treatment), were grown for 6 weeks in a growth room with: (*a*) diffusive ventilation without an ethylene antagonist, (*b*) diffusive ventilation with the ethylene antagonist, silver thiosulphate ($Ag_2S_2O_3$, 0.5 mg l^{-1} of culture medium, i.e. 3·06 μmol Ag l^{-1}), (*c*) convective flow ventilation without an ethylene antagonist, and (*d*) convective flow ventilation with silver thiosulphate (0·5 mg l^{-1} of culture medium). Growth room temperature was 25 $\pm$ 1 °C, PAR approx. 100 μmol m^{-2} s^{-1}, day-length 12 h and relative humidity 40–60 %.

The numbers of leaves produced, and leaf abscission, expressed as a percentage of leaves produced, were recorded for each week of the experiment.

The choice of $Ag_2S_2O_3$ concentration was made after testing plants over a range of concentrations (0·01, 0·05, 0·1, 0·5, 1·0, 2·0, and 5·0 mg l^{-1}). The optimum concentration was 0·5 mg l^{-1}; below this there was some leaf abscission, while above it there was an increasing inhibition of new leaf production and growth.

Effects of various methods of ventilation on ethylene accumulation in culture vessels containing Annona *cuttings*

Culture vessels (30 ml), each containing 5 ml of nutrient medium as above, and an *Annona* cutting (f.wt. 357 $\pm$ 56 mg; leaf area 10·8 $\pm$ 2·6 cm^2) were capped with (*a*) a silicone rubber bung, (*b*) conventional polypropylene film or (*c*) the throughflow ventilation apparatus. Each vessel had a side-arm and Suba-seal for removing gas samples as described previously. Five replicates were prepared per treatment and kept for 48 h at 23 °C, 80 μmol m^{-2} s^{-1} PAR and 37 % RH. Gas samples (500 μl) were removed at intervals and analysed for ethylene concentrations as described for set B.

The production of recalcitrant long-term coconut calloid and application of diffusive or pressure-flow ventilation

When cultured on modified medium (Murashige and Skoog, 1962) containing 0·25 % activated charcoal and 1×10^{-4} M 2,4-D, embryogenic coconut calloid derived from rachillae of the cv. Malayan Dwarf may, on reduction of auxin levels, form somatic embryos and plantlets (Branton and Blake 1983*b*, 1986). However, abnormal development can occur; one of the most common forms being the predominant development of spongy growth resembling the haustorium (or cotyledon) which normally enlarges inside the nut supplying the developing zygotic embryo (Fremond, Ziller and de Nuce de Lamothe, 1966; Branton and Blake 1983*a*). Cultures developing haustorial growth may become embryogenic once again by increasing the 2,4-D level to 4×10^{-4} M for 1 or 2 months. Plantlets have been produced from such recycled material (Blake and Wilson, 1990). Such embryogenic calloid is smooth and shiny (Branton and Blake, 1986). Often, however, a form of long-term calloid develops which continues to divide. This can be subcultured frequently, but is no longer strictly embryogenic, i.e. although a type of calloid continues to form, it can be rougher than true calloid and no longer produces somatic embryoids spontaneously. The use of the pressure-flow ventilating system was tested on such long term coconut calloid in an attempt to return it to embryogenic calloid with embryo and plantlet formation.

Coconut calloid from cv. Malayan Dwarf (Blake and Hornung, 1994) was first cultured on modified Murashige and Skoog medium (Brackpool, Branton, and Blake, 1986) but with BAP omitted and the addition of 2iP at 1×10^{-6} M, 2,4-D at 1×10^{-4} M, and 0·3 % Gelrite used as gelling agent. Each calloid explant (volume approx. 1 ml) was then transferred into a culture tube containing 22 mm depth of modified MS medium, and subjected either to conventional diffusive or throughflow ventilation as described previously. Each treatment contained six replicates. Cultures were transferred or subcultured to fresh medium once a month and kept in the dark at 29 °C.

After 5 months, samples of the calloid from the two types of treatment were wax-embedded, sectioned, stained with haematoxylin and eosin, and examined by light microscopy. Photomicrographs were obtained using an Olympus IMT-2 inverted microscope with camera attachment.

For wax embedding, the specimens were dehydrated using an ethanol series and 'Histo-clear' prior to embedding in Fibrowax (Raymond A. Lamb formulation from MERCK Ltd., Poole, England BH15 1TD).

RESULTS AND DISCUSSION

Rates of throughflow ventilation

As indicated earlier, ventilation rates are at a maximum when the water surface is very close to the membrane of the inflow turret and decrease with time as the water level falls due to evaporation (Fig. 2). This figure also shows that the resistance imposed by the presence of the outflow membrane does not greatly reduce flows.

The results in Table 1 document the throughflow ventilation rates in the assembly with the outflow membrane in position and show that flow rates are relatively unaffected by the presence or absence of agar and a plant in the vessel. However, as expected, no throughflow occurs when the 'inflow turret' is dry, and in this case ventilation is by diffusion via both membranes; this latter case, relative to throughflow ventilation, is explored in the following section (set B).

Effects of different ventilation methods on the rates of ethylene escape from the culture vessel

The results presented in Table 2 indicate that convective throughflow ventilation is the most effective method for

removing ethylene from culture vessels, being more than twice as efficient as the best diffusive method normally used to cover the culture vessels, i.e. polypropylene discs. Cling film was a relatively poor membrane in terms of the diffusive loss of ethylene, while, as expected, the 'Suba-seal' was found to be highly impermeable: here the t_{50} values were more than 130 times larger than the convective flow system. By using hypodermic needles for inflow and outflow tubes, there is the danger of impeded gas flow from condensation droplets forming within them. In the case of the t_{50} experiments, however, the hypodermic needles had no significant effect on the t_{50} values obtained. In retrospect we were concerned also that the silicone rubber tubing used as part of the inflow and outflow paths in the convective flow assembly might have enhanced the rate of ethylene efflux from the culture vessels. Later studies in which less permeable PVC tubing was substituted for the silicone rubber showed that the influence of the silicone rubber on the t_{50} value was not statistically significant.

In Fig. 3, (data for set B), the t_{50} for the removal of ethylene by throughflow ventilation was only 23 min compared to 147 min for the polypropylene disc, and 295 min for the ventilation apparatus functioning diffusively. The reason for the latter functioning less effectively than the polypropylene was no doubt because the membranes were remote from the vessel and the ethylene had to negotiate the tubes and turrets before reaching the mem-

TABLE 2. *The influence of four methods of 'capping' plant tissue culture vessels on the time taken (t_{50}) for 50% of a sample of injected ethylene to be discharged from the system. Culture vessel volumes 30 ml; initial ethylene concentration approx. 70 μl l^{-1}. Relative humidity, 40–60%; T, 25 °C. Data as means ± s.e.* (n = 4)

Method of capping	t_{50} (h)
Suba-seal rubber puncture cap	5587 ± 95
Cling film (PVC)	1313 ± 40
Polypropylene discs + rubber band	94.8 ± 16
Convective flow apparatus (hypodermic needles as inflow and outflow tubes)	43 ± 29
Convective flow apparatus (glass inflow and outflow tubes)	30 ± 15

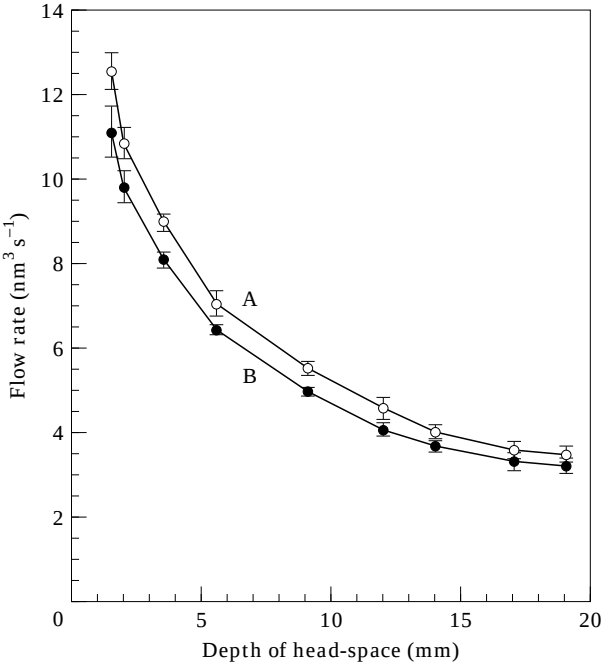

FIG. 2. Showing the effect of head-space depth (distance between membrane and underlying water surface) in the inflow turret on humidity-induced pressure flow ventilation rates: without outflow turret (○) and with outflow turret (●). Membrane pore diameters: inflow turret, 0·03 μm; outflow turret, 0·2 μm. Inside diameters of turrets, 20 mm; ambient temperature 21·5 °C; RH, 36%. Each point is the mean of four replicates; bars = ± s.e.

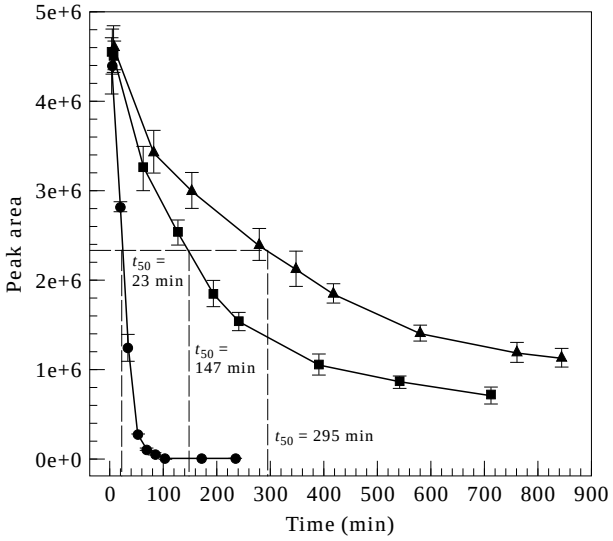

FIG. 3. The influence of various ventilating systems on the time taken (t_{50}) for ethylene at a concentration of 23 μl l^{-1} in N_2 to be discharged from a 30 ml culture vessel: (●) throughflow ventilation apparatus (flow rate 1·0 ml min^{-1}); (■) culture vessel capped with polypropylene membrane; (▲) throughflow ventilation apparatus functioning only as a diffusive ventilating system (inflow and outflow turrets dry). Membrane pore diameters: inflow turret, 0·03 μm; outflow turret, 0·2 μm. Inside diameters of turrets, 20 mm; ambient temperature, 27 °C; RH, 25%. Each point is the mean of four replicates; bars = ± s.e.

TABLE 1. *Ventilation rates through culture vessels. A, B, C: water surface < 2 mm below inflow membrane. Membrane pore diameters: inflow, 0·03 μm; outflow, 0·2 μm. Relative humidity, 17%; T, 25 °C. Data as means ± s.e.* (n = 3)

Culture Vessels	Inflow Turrent	Flow rates (nm^3 s^{-1})
A empty	humidified	17·03 ± 0·06
B + 5 ml culture medium	humidified	17·47 ± 0·22
C + 5 ml culture medium + plant	humidified	17·49 ± 0·22
D empty	dry	0

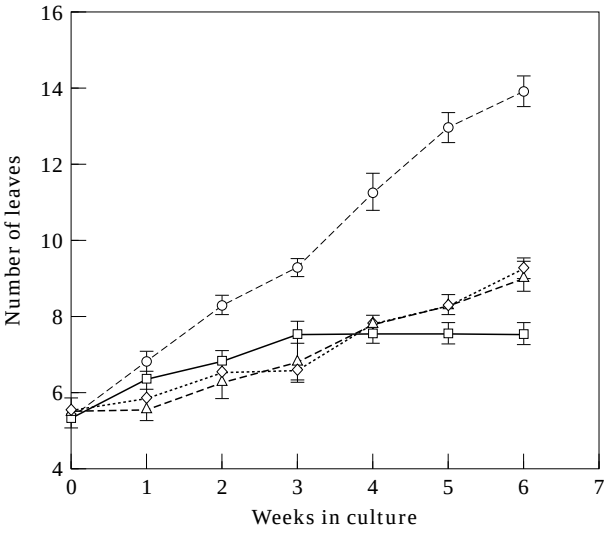

FIG. 4. Leaf production of *Annona squamosa* cuttings: the effects of the pressure-flow or the diffusive ventilating systems, with and without the ethylene antagonist silver thiosulphate (STS): 0·5 mg l^{-1}, i.e. 3·06 μmol Ag l^{-1}, in the culture medium. Means and standard errors of four replicates per treatment. Convective flow ventilation (○); convective flow ventilation + STS (△); diffusive ventilation (□); diffusive ventilation + STS (◇).

branes. Here the t_{50} for the throughflow ventilation compared to that with set A (Table 2) can be accounted for by a narrower head space in the in-flow turret and the lower RH of set B.

Effects on Annona *of different ventilation methods, with or without an ethylene antagonist*

After 6 weeks, explants grown in convective ventilation without $Ag_2S_2O_3$ in the medium, had produced at least 1·5 times more leaves than those in any other treatment (Fig. 4), and twice the number produced with diffusive ventilation without $Ag_2S_2O_3$. The leaves were larger with convective ventilation than in the other treatments, (results not shown).

For plants grown in diffusive ventilation and lacking $Ag_2S_2O_3$, the number of dropped leaves was the greatest up to week 5, but at 6 weeks the number was similar to that of the treatment with throughflow ventilation, (Fig. 5A). However, in the former treatment, where there were few leaves, almost all (98%) had dropped, whereas in the latter where leaves were more numerous because of a greater production, only approx. 50% had dropped (Fig.5B).

In treatments with either convective or diffusive ventilation with $Ag_2S_2O_3$ in the medium, the numbers of leaves produced during the experimental period were similar, and lower than those produced in convective ventilation without $Ag_2S_2O_3$ (Fig.4). Also, the numbers of leaves abscinded (Fig. 5A), and the percentages of abscissions in the two former treatments, were lower than in the other two treatments (Fig. 5B). Toxic effects of $Ag_2S_2O_3$ have been reported (Wang and Dunlap, 1990) and though smaller percentages of abscission were found in both treatments

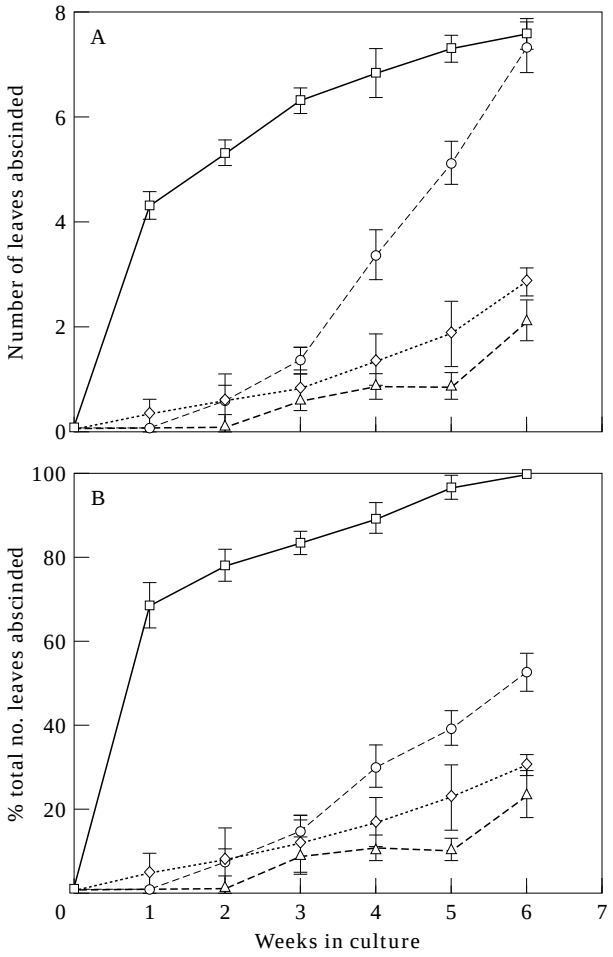

FIG. 5. *Annona squamosa* cuttings: the effects of the pressure-flow or diffusive ventilating systems, with and without the ethylene antagonist silver thiosulphate (STS) in the culture medium on (A) leaf fall, and (B) per cent leaf abscission [(number of leaves abscinded/total number of leaves) × 100]. Diffusive ventilation (□); diffusive ventilation + STS (◇); convective flow ventilation (○); convective flow ventilation + STS (△). Means and standard errors of four replicates per treament. STS at 0·5 mg l^{-1}, i.e. 3·06 μmol Ag l^{-1}.

with $Ag_2S_2O_3$, it seems possible that a toxic effect of that compound had influenced the number of leaves produced (Fig. 4).

Effects of various methods of ventilation on ethylene accumulation in culture vessels containing Annona *cuttings*

As expected, ethylene accumulated to a far greater extent in the sealed vessel compared to the ventilated ones, with mean concentrations amounting to approx. 1·9 μl l^{-1} in the former after 48 h, whereas with polypropylene capping the value was > 0·2 μl l^{-1}, and with throughflow ventilation it was negligible (Fig. 6). *Annona* has been found to be very sensitive to ethylene (Lemos and Blake, 1994), and it seems possible that the accumulation of the gas in vessels capped with polypropylene might have been sufficient to contribute to the greater incidence of leaf abscission (Fig. 5) and the smaller numbers of leaves produced (Fig. 4) compared to

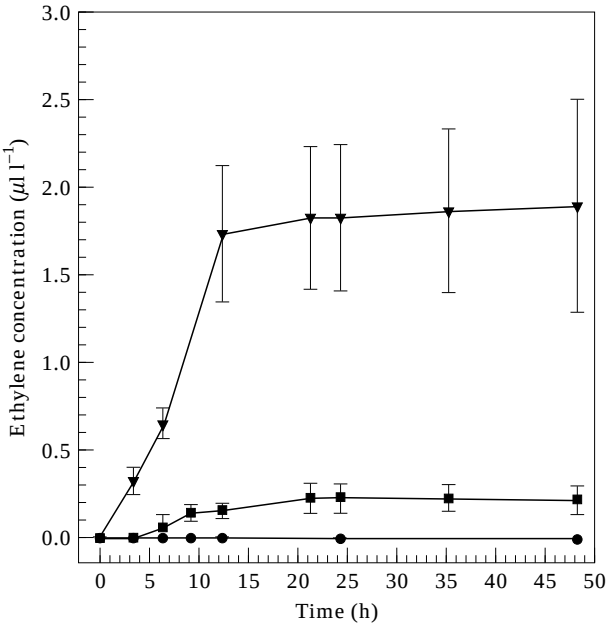

FIG. 6. Effect of culture vessel ventilating type on ethylene accumulation from *Annona squamosa* L. cuttings. Points show means of five replicates ± s.e. Leaf area of cuttings = 10·8 ± 2·6 cm²; total fresh weight = 357 ± 56 mg; growth room temperature, 23 °C; RH, 37%; 30 ml culture vessels were used with 5 ml of medium. (▼) Sealed; (■) polyproplene membrane; (●) throughflow ventilation.

the plants grown in throughflow ventilation. This would accord with the effects of STS.

Ethylene accumulation in the polypropylene-capped vessels (Fig. 6) occurred only after 3 h and we believe may have been augmented by the development of condensation on the underside of the membrane. In our experience this is of common occurrence, and may be a major draw back of such systems. In wider 60 ml vessels the delay in ethylene accumulation was about 10 hours (data not shown) and probably accords with a slower development of condensation.

Effects of diffusive or convective ventilation on coconut calloid

After 20 weeks, the long-term calloid in the convective flow treatments produced a fine, more convoluted calloid, compared to the rougher, more haustorial, type of growth produced in diffusive ventilation. Histological sections showed that the calloid which had received convective ventilation was composed of smaller cells than the calloid subject to diffusive ventilation, and had a distinct epidermal layer, giving it a smooth, shiny appearance; in certain regions a distinct hypodermal epidermal layer was also present. This calloid also produced a profusion of clearly-defined sub-epidermal meristematic nodules, (Fig. 7A ,C, E). In contrast, the calloid which had received only diffusive ventilation was composed of larger cells, the epidermis was poorly defined, and there was no obvious hypodermis; here, there were fewer and less discrete sub-epidermal meristematic nodules, (Fig. 7B, D, F). Also, in this callus, cells containing raphides were observed; these were absent from the calloid which had received throughflow ventilation. In both types of calloid, vascular elements with helical and scalariform thickenings were present.

FINAL DISCUSSION

Using t_{50}s as indicators, the throughflow method of ventilation at a relative humidity of 25% has been shown to be more than six times more efficient in facilitating the removal of ethylene from the culture vessels, compared to the most efficient conventional diffusive method investigated, i.e. via polypropylene discs (Fig.3). Even at a higher RH of 60%, the throughflow ventilation was > 2·4 times more efficient (Table 2). We have recent evidence (Zobayed, 1996) that this pressurized throughflow ventilation is similarly more efficient in sustaining CO_2 levels well above the compensation point during the day, and O_2 levels at night within the culture vessels. In terms of reducing ethylene accumulation, however, the throughflow ventilation can be regarded as very much more efficient than the polypropylene diffusive system (Fig. 6).

The results also indicate that, compared to ventilation via polypropylene discs, convective throughflow ventilation increased leaf production and leaf growth in *Annona*; because of the increased leaf production the percentage leaf abscission was lower with the convective flow system. Also, coconut calloid was found (*a*) to contain more meristematic centres in the presence of convective-flow ventilation, and (*b*) to develop a more definite epidermal layer and a smoother surface of the nodules. Thus, this long-term calloid resembled newly formed calloid which is capable of somatic embryo and plantlet formation. The convective flow ventilation appeared to have assisted, at least partly, in moving towards the aim of obtaining vegetative propagation from 'long-term' coconut calloid.

We suggest that the beneficial effects on the *Annona* plants were probably due to the more effective removal of ethylene in the convective flow assemblies, especially since the plants similarly responded to an ethylene antagonist. However, the plants may also have benefitted from higher oxygen concentrations at night and a higher CO_2 supply during the day induced by throughflow ventilation. Similarly, the coconut calloid may have also benefitted from the removal of ethylene and from increased oxygen concentrations and the more efficient removal of CO_2.

The throughflow ventilation system described here appears to have the following advantages: (*a*) it is a simple system requiring no pumps, gas cylinders etc., (*b*) it prevents the entry of contaminants such as bacteria and fungal spores, (*c*) it will reduce CO_2 depletion in the light and its accumulation in the dark, (*d*) it will ensure near-atmospheric levels of O_2 throughout the day and night, (*e*) it flushes the system free from potentially toxic gases, e.g ethylene, and (*e*) it can eliminate the need for the use of ethylene absorbents or antagonists. With this type of system, CO_2-enrichment could easily be applied during the day for increasing photosynthesis.

While Jackson *et al.* (1991) acknowledge that the problem of ethylene accumulation can be lessened by the use of

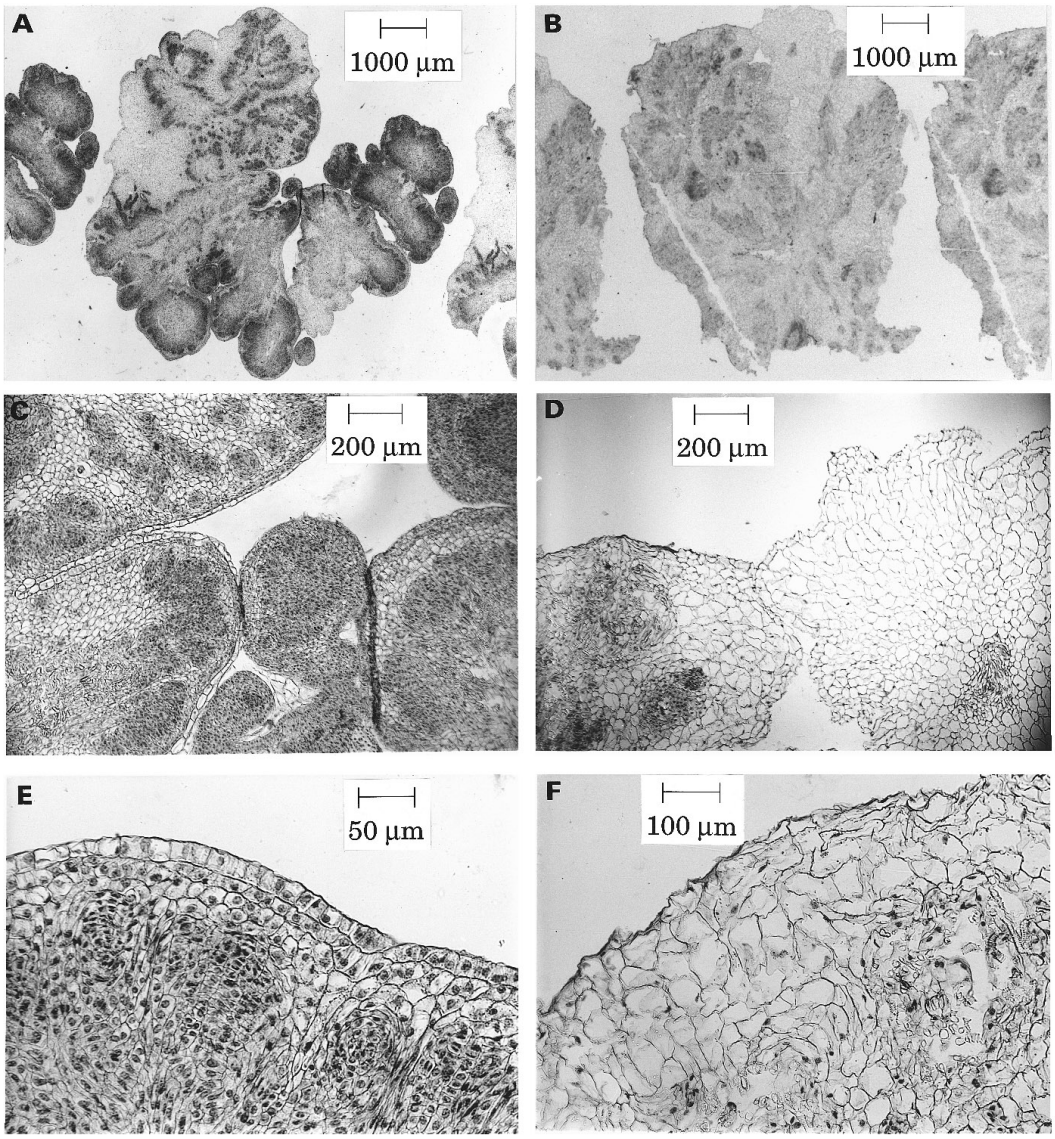

FIG. 7. Sections of wax-embedded, long-term coconut calloid of cv. Malayan Dwarf which had been grown for 20 weeks in either a sterile, convective flow ventilation system (A, C, E), or a conventional culture system relying on gas diffusion through polypropylene film (B, D, F). Details as follows. A, Note the markedly convoluted shape, and profusion of discrete sub-epidermal meristematic nodules. B, Note the non-convoluted shape and fewer and less discrete meristematic nodules. C, Note the distinct well differentiated epidermal and, in places, hypodermal layers, small cells and discrete meristematic nodules. D, Note the larger cells, and less well defined epidermal layer. E, Showing similar features to (C). F, Showing similar features to (D).

larger culture vessels, the system described here makes possible the use of smaller vessels. A further possible advantage of the system is that the aerating gases are humidified, and this should help to reduce losses of water vapour from both plants and medium. Sallanon and Maziere (1992), found that low vessel and growth room humidities, by inducing high transpiration rates, produced large changes in the growth and morphology of rose plants, with decreased multiplication rates, short shoots and the cessation of the growth of the upper axillary buds. These authors concluded that the control of humidity during the multiplication phase is as important as medium composition, light and temperature parameters in the management of the plants. On the other hand, Debergh *et al.* (1992) found that the quality of *in vitro* cultured plants such as *Rosa multiflora* and *Gerbera jamesonii* could be improved by lowering the humidity of gases in the header space, compared to that developed in a closed container. They concluded that vitrification was correlated with too high a humidity in the head space. As described here the humidity-induced pressurized ventilating system results in an RH of 88–94% in the culture vessel. However, using larger membranes it is possible to create a range of controlled humidities in the culture tube of from < 70 to $> 90\%$ RH (Armstrong, Armstrong and Zobayed, unpubl. res.).

A disadvantage of the humidity-induced pressure flow

system, however, is the need to adjust daily the level of water in turret A, to maintain a high flow. The use of a constant head device connected to turret A could reduce the frequency of these adjustments. Also, if desired, much larger inflow and outflow chambers, with large Nuclepore filter membranes could be employed to create more rapid ventilation in culture vessels containing several plants, or to supply fresh air to several small vessels in parallel. We have recently developed a system combining the inflow and outflow turrets in one unit, which sustains effective humidification for the inflow of air for at least a week without attention (Armstrong, Armstrong and Zobayed, unpubl. res.).

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LITERATURE CITED

Adkins SW. 1992. Cereal callus cultures: control of headspace gases can optimise the conditions for callus proliferation. *Australian Journal of Botany* **40**: 737–749.

Armstrong J. 1992. *Pathways and mechanisms of aeration in* Phragmites australis. PhD Thesis, University of Hull, U.K.

Armstrong J, Armstrong W. 1990. Light-enhanced convective through-flow increases oxygenation in rhizomes and rhizosphere of *Phragmites australis* (Cav.) Trin. ex Steud. *New Phytologist* **114**: 121–128.

Armstrong J, Armstrong W. 1994. A physical model involving nuclepore membranes to investigate the mechanism of humidity-induced convection in *Phragmites australis*. *Proceedings of the Royal Society of Edinburgh* **102B**: 529–540

Armstrong W, Armstrong J, Beckett PM. 1996*a*. Pressurised aeration in wetland macrophytes: some theoretical aspects of humidity-induced convection and thermal transpiration. *Folia Geobotanica et Phytotaxonomica* **31**: 25–36.

Armstrong W, Armstrong J, Beckett PM. 1996*b*. Pressurised ventilation in emergent macrophytes: the mechanism and mathematical modelling of humidity-induced convection. *Aquatic Botany* (in press).

Armstrong J, Armstrong W. 1996*c*. *Ventilation apparatus and system*. University of Hull UK Patent No. GB227505B. The Patent Office, London. Publication number (August 1994) 2275052A.

Armstrong W, Armstrong J, Beckett PM, Justin SHFW. 1990. Convective gas-flows in wetland plant aeration. In: Jackson MB, Davies DD, Lambers H, eds. *Plant life under oxygen stress*. The Hague: SPB Academic Publishing bv, 283–302.

Blake J, Hornung R. 1994. Somatic embryogenesis in coconut. In: Jain SM, ed. *Somatic embryogenesis in woody plants*. Dordrecht, The Netherlands: (in press).

Blake J, Wilson N. 1990. Problems of somatic embryogenesis in coconut (*Cocos nucifera* L.). In: *Abstracts of the VIIth International Congress of Plant Tissue and Cell Culture*, Amsterdam 24–29 June 1990. International Association of Plant Tissue Culture. Abstract B4–19, 245.

Brackpool AL, Branton RL, Blake J. 1986. Regeneration in palms. In: Vasil IK, ed. *Cell culture and somatic cell genetics of plants*. London: Academic Press, 207–222.

Branton RL, Blake J. 1983*a*. Development of organised structures in callus derived from explants of *Cocos nuciflora* L. *Annals of Botany* **52**: 673–678.

Branton RL, Blake J. 1983*b*. A lovely clone of coconuts. *New Scientist* **98**: 554–557.

Branton RL, Blake J. 1986. Clonal propagation of coconut palm. In: Pushparajah E, Chew Poh Soon, eds. *Cocoa and coconuts: progress and outlook*. Kuala Lumpur: Incorporated Society of Planters, 771–780.

Brix H, Sorrell BK, Orr PT. 1992. Internal pressurisation and convective gas-flow in some emergent freshwater macrophytes. *Limnology and Oceanography* **37**: 1420–1433.

Buddendorf-Joosten JMC, Woltering EJ. 1996. Controlling the gaseous composition *in vitro*–description of a flow system and effects of the different gaseous components on *in vitro* growth of potato plantlets. *Scientia Horticulturae* **65**: 11–23.

Dacey J. W. H. 1981. Pressurised ventilation in the yellow water-lily. *Ecology* **62**: 1137–1147.

Debergh PC, De Meester J, De Riek J, Gillis S, Huylenbroeck J van. 1992. Ecological and physiological aspects of tissue-cultured plants. *Acta Botanica Neerlandica* **41**: 417–423.

De Proft MP, Maene LJ, Debergh PC. 1985. Carbon dioxide and ethylene evolution in the culture atmosphere of *Magnolia* cultured *in vitro*. *Physiologia Plantarum* **65**: 375–379.

Fremond Y, Ziller R, de Nuce de Lamothe M. 1966. *The coconut palm*. International Potash Institute (Publ).

Jackson MB, Abbott AJ, Belcher AR, Hall KC. 1987. Gas exchange in plant tissue cultures. In: Jackson MB, Mantell S, Blake J, eds. *Advances in the chemical manipulation of plant tissue cultures*. BPGRG Monograph 16. Bristol: British Plant Growth Regulator Group, 57–71.

Jackson MB, Abbott AJ, Belcher AR, Hall KC, Butler R, Cameron J. 1991. Ventilation in plant tissue culture and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. *Annals of Botany* **67**: 229–237.

Jackson MB, Belcher AR, Brain P. 1994. Measuring shortcomings in tissue culture aeration and their consequences for explant development. In: Lumsden PJ, Nicholas JR, Davies WJ, eds. *Physiology, growth and development of plants in culture*. Dordrecht, The Netherlands: Kluwer Academic, 191–203.

Kozai T, Kitaya Y, Kubota C. 1995. *Collected papers on environmental control in micropropagation*, Vol. 3 (1994–1995). Genhua Niu, ed. Laboratory of Environmental Control Engineering, Faculty of Horticulture, Chiba University, Chiba 271, Japan.

Kozai T, Kubota C, Nakayama M. 1989. Net photosynthetic rates of plantlets *in vitro* under natural and forced ventilation conditions. *Annual Meeting, Japanese Society of Horticultural Science*, 250–251.

Lemos EEP, Blake J. 1994. Leaf abscission in micropropagated sugar apple (*Annona squamosa* L.). In: Lumsden PJ, Nicholas JR, Davies WJ, eds. *Physiology, growth and development of plants in culture*. Dordrecht, The Netherlands: Kluwer Academic, 227–232.

Murashige T, Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–497.

Righetti B, Facini O. 1992. Headspace gas composition in four *Prunus avium* cultivars with differing photosynthetic capabilities. In vitro *cellular developmental biology*, (Tissue Culture Association) **28P**: 179–182.

Righetti B, Magnanini E, Infante R. 1990. Ethylene, ethanol, acetaldehyde and carbon dioxide release by *Prunus avium* shoot cultures. *Physiologia Plantarum* **78**: 507–510.

Righetti B, Magnanini E, Maccaferri M. 1987. Ethylene and other volatile substances produced by *in vitro* cultured *Prunus avium*. *Acta Hortanica* **227**: 402–404.

Rossetto M, Dixon KW, Bunn E. 1992. Aeration: a simple method of control of vitrification and improved *in vitro* culture of rare Australian plants. In vitro *cellular developmental biology*, (Tissue Culture Association) **28P**: 192–196.

Sallanon H, Maziere Y. 1992. Influence of growth room and vessel humidity on the *in vitro* development of rose plants. *Plant Cell, Tissue and Organ Culture* **30**: 121–125.

Thomas D, Des S, Murashige T. 1979. Volatile emissions of plant tissue cultures. I. Identification of the major components. *In Vitro* **15**: 654–658.

Wang Y-T, Dunlap JR. 1990. Leaf abscission in *Radermachera sinica* in response to ethylene and silver thiosulphate. *Horticultural Science* **25**: 233.

Woltering EJ. 1986. Ethylene and carbon dioxide accumulation within various tissue culture systems. *Acta Botanica Neerlandica* **35**: 50 (Abstract).

Yue D, Gosselin A, Desjardins Y. 1993. Effects of forced ventilation at different relative humidities on growth, photosynthesis and transpiration of geranium plantlets *in vitro*. *Canadian Journal of Plant Science* **73**: 249–256.

Zobayed, SMA. 1996. *The use of a natural pressorized forced ventilation in plant micropropagation*. PhD Thesis, University of Hull.